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14. ABSTRACT

Aim 1: We report a three-fold increase in lung metastasis and a 50% increase in the incidence of bone metastasis in the arthritic (SKG) mice compared to non-arthritic (Balb/c) mice. Interestingly, we found that the metastatic breast tumors augment the severity of arthritis resulting in a vicious cycle that increases both bone destruction and increased metastasis. Enhanced neutrophilic and granulocytic infiltration in lungs and bone of the arthritic mice and subsequent increase in circulating levels of pro-inflammatory cytokines, such as IL-17, M-CSF, IL-6, VEGF, and TNF-α were the underlying factors contributing to the increased metastasis. **Aim 2:** In the PyV MT mice that develop spontaneous breast cancer, collagen induced-arthritis significantly enhanced the primary tumor burden and the incidence of bone and lung metastasis. In these arthritic PyV MT mice, increase in the pro-inflammatory cytokines was followed by a significant reduction in the numbers of natural killer (NK) cells and a significant increase in the myeloid suppressor cells (MSCs) within the tumor microenvironment. **Aim 3:** The efficacy of anti-IL-17 antibody treatment in combination with COX-2 inhibitor was more efficacious than either treatment alone in the prevention of bone and lung metastasis in the arthritic (SKG) mice bearing the metastatic breast tumor and **Aim 4:** Analysis of archival cases of breast cancer patients from 1996 to 2007, revealed a trend towards a positive correlation between metastasis and AA, however, due to diverse treatment regimen, statistical significance was not reached.

15. SUBJECT TERMS
Arhritis, Breast Cancer, Bone metastasis, Lung metastasis, Inflammation

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Title: Study the influence of arthritis on breast cancer associated bone metastasis

INTRODUCTION: Metastasis is regulated not only by intrinsic genetic changes in malignant cells, but also by the microenvironment. Several studies have demonstrated that sites of chronic inflammation are often associated with the establishment and growth of various malignancies (1). A common inflammatory condition in humans is autoimmune arthritis (AA) that causes inflammation and deformity of the joints. Other systemic effects associated with arthritis include increased cellular infiltration and inflammation of the lungs (2). Several studies have also reported statistically significant risk ratios between AA and various malignancies including breast, lung, hematopoietic, nonmelanotic skin, kidney, and colon **(3, 4**) **(5,6).** Despite this knowledge available for a decade, it has never been questioned if a site of chronic inflammation linked to AA creates a milieu that attracts tumor cells to home and grow in the inflamed bones and lungs which are frequent sites of breast cancer metastasis (**7)**. The preference of breast cancer cells to grow in the bone and lung is underscored by the fact that 65-75% of patients with advanced disease develop metastases in these organs. Yet, it is not known why and how breast cancer cells prefer to colonize these organs. There are no methods to predict the risk of breast cancer-associated metastasis and current treatments have notable limitations.

Original Hypothesis: Chronic inflammatory milieu and osteoclastic bone resorption caused by autoimmune arthritis may influence the recruitment, retention, and proliferation of tumor cells in the bone and promote metastasis. **Although we set out to only study bone metastasis, we discovered that AA has a significant effect on lung metastasis as well and therefore we have included data on bone and lung metastasis.**

BODY:

AIM 1: To determine the incidence and frequency of bone metastasis in a spontaneous model of autoimmune arthritis injected with syngeneic metastatic or non metastatic breast cancer cells (4T1 or TUBO cell lines respectively) in the mammary fat pad. The data from this aim has been submitted for publication to Nature Medicine. A pdf of the manuscript is attached (appendix 1).

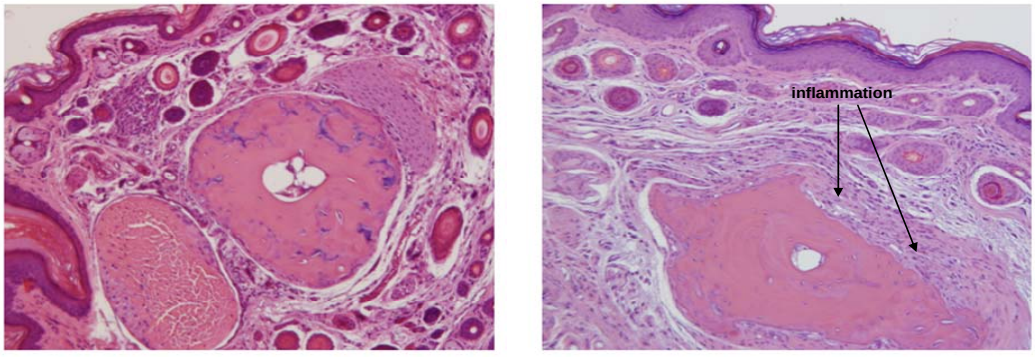

Figure 1: PyV MT mice injected with CII developed arthritis by 21 – 23 weeks of age.

AIM 2: To determine if bone metastasis develops in mouse models of spontaneous breast cancer.

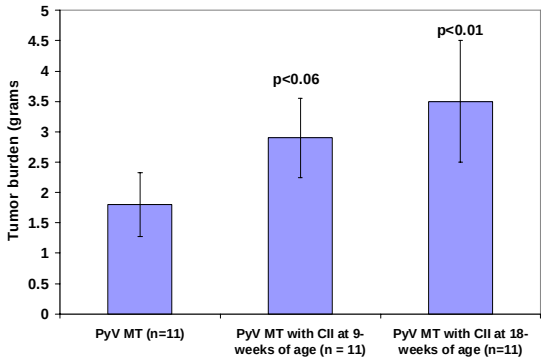

Figure 2: Increase in primary tumor burden in arthritic PyV MT mice as compared to the non-arthritic PyV MT mice.

The PyV MT mice that develop spontaneous cancer are injected with 2mgs/ml Type II collagen (CII) to induce arthritis at two time points: when the mice are 9 weeks in pre-metastatic condition and when the mice are 18 weeks with primary tumors large enough and metastasis is expected to occur. Emulsified CII in CFA was injected intradermally (i.d) in the skin of the tail at approximately 1.5 cm distal to the base of the tail. Approximately 60% of the mice injected with CII developed inflammation in the joints. Between 21 and 23 weeks, mice were sacrificed and arthritic limbs were represented by high cellular infiltration in the bones (Figure 1). This inflammation resulted in an increase in the primary tumor burden in the arthritic PyV MT mice versus the control PyV MT mice regardless of whether arthritis was induced at 9 or 18 weeks of age (Figure 2). This was further substantiated by the increased presence of proliferating cells within the tumor of the arthritic PyV MT mice compared to non-arthritic PyV MT mice (Figure 3). This was determined by proliferating cell nuclear antigen (PCNA) staining of the tumor sections. When bone marrow of these mice was examined, we observed a significant increase in circulating epithelial tumor cells in the arthritic PyV MT mice versus non-arthritic PyV MT mice. Although bone lesions were not observed in the PyV MT mice, the increased pancytokerain+ cells in the marrow is a clear indication of increased tumor cell recruitment to the bone. This may be because the mice cannot be aged beyond 23 weeks, as the primary tumor burden reaches 10% of the body weight and due to IACUC regulations; the mice have to sacrificed at that time. In the future, we propose to surgically remove the primary tumor and allow the animals age to follow bone lesions. With regard to lung metastasis, we observed a significant increase in the incidence of metastasis in arthritic (27% in 9-week CII mice and 36% in 18-week CII mice) versus the non-arthritic PyV MT mice (0%) (Figure 5). Most

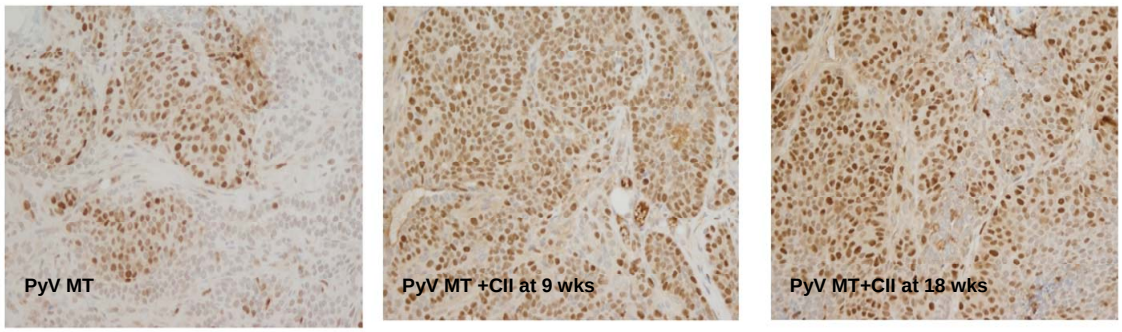

Figure 3: Highly proliferative PCNA$^+$ cells in the arthritic PyV MT versus non-arthritic PyV MT mammary gland tumor.

importantly, the arthritic PyV MT mice have lower survival (21 weeks) compared to the non-arthritic PyV MT (26 weeks) mice.

Mechanistically, several factors were discovered to be critical for the increased primary tumor and metastasis. First, there were increased pro-inflammatory factors such as COX-2, PGE2, VegF, and CD31 in the arthritic bones of PyV MT mice as well as in the primary tumor along with higher cellular infiltration in the bones and lungs. Second, the level of circulating IL-17 was increased in the arthritic PyV MT mice. Third, there was a significant decrease in NK cells and significant increase myeloid suppressor ($CD11b^{+}/GR1^{+}$) cells in the arthritic PyV MT versus non-arthritic PyV MT mice. Activated NK cells are potent sources of immune modulatory cytokines that directly aid in the elimination of tumor cells and also indirectly augment a developing adaptive immune response against tumor growth. Decrease in NK cells observed may contribute to the low survival of the arthritic PyV MT mice.

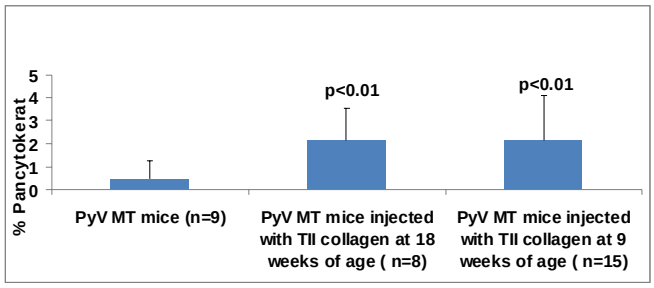

Figure 4: Increased pancytokeratin^{+} cells in the arthritic versus non-arthritic PyV MT mice.

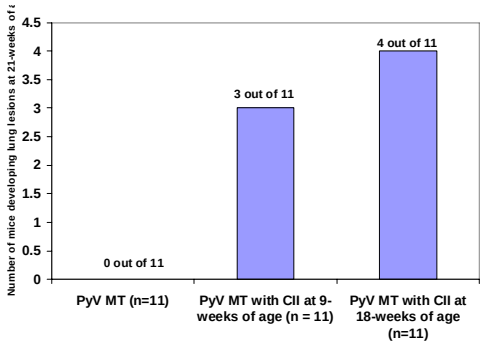

Figure 5: Increased lung metastasis in arthritic versus the non-arthritic PyV MT mice. Number of mice that developed lung metastatis at 21-weeks of age.

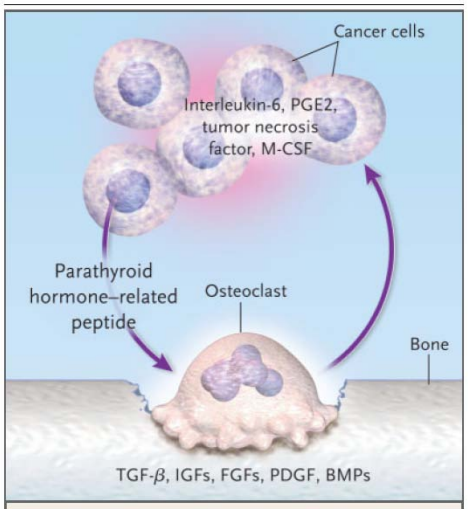

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Figure 6: The vicious circle of osteolytic metastasis

Concluding Remarks and Future Direction for Aims 1 and 2: A schematic representation of a proposed vicious cycle of osteolytic metastasis is illustrated in Figure 6. We have been able to test this phenomenon directly using the SKG and the PyV MT models. These studies warrant further investigation into understanding the key mechanism that drives increased breast cancer-associated metastasis in arthritic mice. One suggested mechanism (that was previously reported is schematically illustrated in Figure 7) which can be tested for the first time in our models.

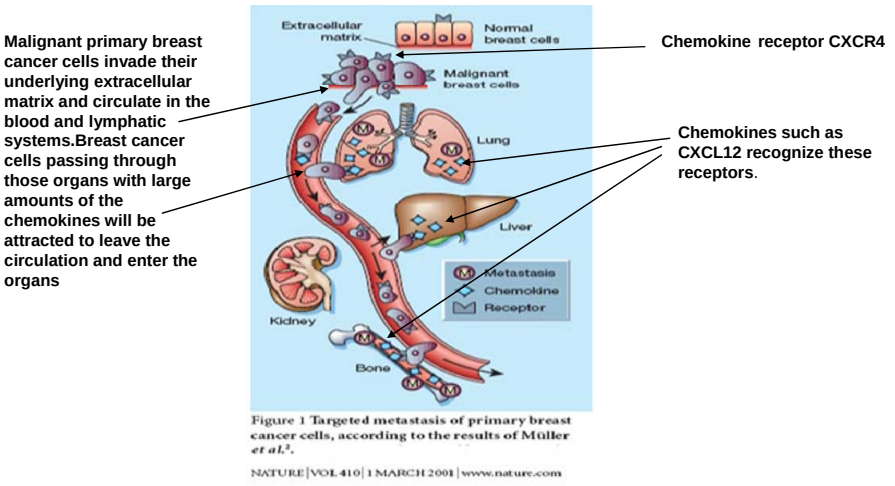

Figure 7: Proposed mechanism for targeted metastasis of primary breast cancer cells

AIM 3: The efficacy of anti-IL-17 antibody treatment in combination with COX-2 inhibitor was more efficacious than either treatment alone in the prevention of bone and lung metastasis in the arthritic (SKG) mice bearing the metastatic breast tumor. The treatment schedule and dose was described in the proposal. The data also suggest that these two agents were not able to completely prevent metastasis from occurring. Other factors have to be evaluated. The primary tumor was removed before treatment was started. Similar results were observed with the EP2 receptor antagonist as celecoxib (data not shown).

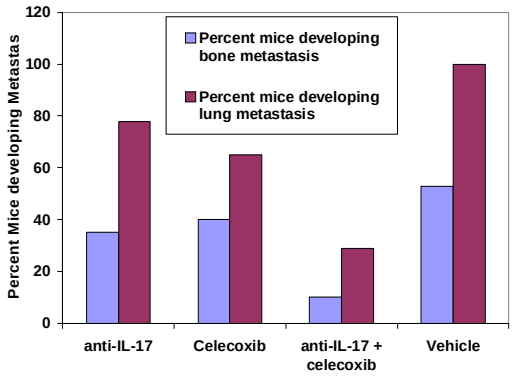

Figure 8. The combination of celecoxib + anti-IL-17 was more effective in reducing metastasis in SKG mice challenged with 4T1 tumors. N=10 mice per group.

AIM 4: Analysis of archival cases of breast cancer patients from 1996 to 2007, revealed a trend towards a positive correlation between metastasis and AA, however, due to diverse treatment regimen, statistical significance was not reached. Mayo Clinic Arizona Cancer Registry was used to sort analytical female breast malignancies from 1996-2007. Total of three thousand seven hundred and eighty patient records were evaluated. Out of these there were approximately 567 had metastatic disease in various sites including multiple sites. 63 patients had bone metastasis and only 13 were alive beyond one year. Fifty patients with bone metastasis died within one year of diagnosis. Almost all patients suffered from some form of AA. Since many of the non-metastatic breast cancer patients also had AA, the statistical analysis did not reveal a clear correlation. The data is however extremely enticing and many more patients need to be evaluated in the future.

KEY RESEARCH ACCOMPLISHMENTS:

- Significantly increased bone and lung metastasis is observed in mice with AA
- Significant increase in the severity of arthritis in mice bearing metastatic tumors
- Key factors that are implicated include: high cellular (neutrophilic and granulocytic) infiltration in the site of metastasis; increased circulating levels of IL-17, IL-6, TNF-α, and VegF in the arthritic mice; high pro-inflammatory COX-2 and CD31 expression in the arthritic mice; decreased levels of activated NK cells; and increased levels of myeloid suppressor cells in the arthritic mice
- A strong statistical correlation exists between bone inflammation and lung and bone metastasis
- Combination of IL-17 antibody and celecoxib is more effective in preventing metastasis
- Patients with bone metastasis tend to have some form of AA.
- Future funding is being seeked to continue this important study from NIH and DOD

REPORTABLE OUTCOMES:

- SKG mice data: Manuscript submitted to Nature Medicine
- PyV MT mice data: Manuscript to be submitted to Cancer Research
- Treatment data: Manusrcipt in preparation
- Human Data: Manuscript in preparation

- Presentation of the data at the Era of Hope Meeting and at The immunology Meeting (2008)

- AA affects both lung and bone metastasis associated with breast cancer

CONCLUSION:

We have been able to directly test using the SKG and the PyV MT models that a vicious circle of osteolytic metastasis occurs in which both the severity of arthritis and bone destruction is enhanced by metastatic tumor cells and that the arthritic miluei attracts and retains metastatic tumor cells in the site of inflammation. In addition the arthritic milieu increases the primary tumor burden. The two diseases feed of each other. The results warrant further investigation into understanding the key mechanism that drives increased breast cancer-associated metastasis in arthritic mice. One suggested mechanism is the CXCR4 and CXCL-12 interaction which can be tested for the first time in our models. The combination of celecoxib and anti-IL-17 shows impressive results but is not enough to prevent metastasis from occurring. Other factors that we identified especially IL-6, and VegF may play critical role. Whether a combination therapy can be developed that target multiple cytokines for preventing metastasis remains to be investigated. More human data has to be analyzed from multiple cancer centers to determine a statistical correlation.

REFERENCES:

1. deVisser,K.E.,and M.Coussens. 2006. The inflammatory tumor microenvironment and its impact on cancer development. *Contrib Microbiol.* 13:118-137**.**
2. Majithia,V., Geraci S.A.2007. Rheumatoid arthritis: diagnosis and management. *Am. J. Med.* 120 : 936–9
3. Franklin, J., M. Lunt, D. Bunn, D. Symmons, and A. Silman. 2007. Influence of inflammatory polyarthritis on cancer incidence and survival: results from a community-based prospective study. *Arthritis Rheum.* 56:790-798.
4. Mellemkjaer, L., M.S. Linet, G.Gridley, M.Frisch, H. Moller, and J.H. Olsen. 1996. Rheumatoid arthritis and cancer risk.. *Eur J Cancer*. 32: 1753-1757.
5. Askling,J., C.M. Fored, L. Brandt , E. Baecklund, L. Bertilsson, N. Feltelius et al. 2005. Risks of solid cancers in patients with rheumatoid arthritis and after treatment with tumor necrosis factor antagonists. *Ann Rheum Dis* . 64:1421-1426.
6. Askling, J., C.M.Fored, E. Baecklund, L.Brandt , C. Backlin , A. Ekbom , et al. 2005. Haematopoietic malignancies in rheumatoid arthritis: lymphoma risk and characteristics after exposure to tumor necrosis factor antagonists. *Ann Rheum Dis* 64:1414-1420.
7. Andy J. Minn., P.G. Gupta, P. M. Siegel, P. D. Bos, W. Shu, D. Giri, A. Viale, A. B. Olshen, W. L. Gerald and J.Massague.2005. Genes that mediate breast cancer metastasis to lung. *Nature* 436: 518-524.

Breast Cancer Associated Metastasis is Significantly Increased in a Model of Autoimmune Arthritis

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ABSTRACT

Chronic inflammation is known to play a role in cancer initiation, promotion,

and metastasis, however, the mechanism by which inflammation promotes metastasis is still unclear. We evaluated if chronic inflammation induced by autoimmune arthritis may contribute to increased breast cancer-associated metastasis. We report a three-fold increase in lung metastasis and a significant increase in the incidence of bone metastasis in the pro-arthritic mice compared to control mice. The metastatic breast tumors in turn augment the severity of arthritis resulting in a vicious cycle that increases both bone destruction and increased metastasis. Enhanced neutrophilic and granulocytic infiltration in lungs and bone of the pro-arthritic mice and subsequent increase in circulating levels of proinflammatory cytokines, such as IL-17, IL-6, VEGF, and TNF-α were the underlying factors contributing to the increased metastasis. The data clearly has important clinical implications for patients diagnosed with metastatic breast cancer.

INTRODUCTION

Metastasis is regulated not only by intrinsic genetic changes in malignant cells, but also by the microenvironment. Several studies have demonstrated that sites of chronic inflammation are often associated with the establishment and growth of various malignancies (1). A common inflammatory condition in humans is autoimmune arthritis that causes inflammation and deformity of the joints. Other systemic effects associated with arthritis include increased cellular infiltration and inflammation of the lungs and blood vessels (vasculitis), and weakening of the bones (osteoporosis). Although arthritis and cancer are different diseases, some of the underlying processes that contribute to the disorders of the joints and connective tissue that characterize arthritis also affect cancer progression and metastasis. In addition, the immune system appears to play an overseer's role in both diseases reviewed by (**2).** The most striking link between the two diseases came from a long-term community-based prospective study of the influence of inflammatory polyarthritis (IP) in cancer incidence and survival **(3**). The authors reported that inflammatory arthritis increases the risk of dying from cancer (at least double the risk of general population). Several studies have also reported statistically significant risk ratios between arthritis and various malignancies including breast, lung, hematopoietic, nonmelanotic skin, kidney, and colon **(4**) **(5,6).**

Despite this knowledge available for a decade, there has been minimal research linking arthritis with metastatic breast cancer, especially since both diseases are highly prevalent in women. It has never been questioned if a site of chronic inflammation linked to arthritis creates a milieu that attracts tumor cells to home and grow in the inflamed site. The lungs and bones are frequent sites of breast cancer metastasis (**7)**. The preference of

breast cancer cells to grow in the bone and lung is underscored by the fact that 65-75% of patients with advanced disease develop bone or lung metastases. Yet, it is not known why and how breast cancer cells prefer to colonize these organs. There are no methods to predict the risk of breast cancer-associated metastasis and current treatments have notable limitations. We hypothesize that chronic inflammatory milieu and osteoclastic bone resorption caused by autoimmune arthritis and the lung inflammation associated with arthritis may influence the recruitment, retention, and proliferation of tumor cells in the bone and lungs.

In this study, we determined if chronic inflammation in the bone and other internal organs (such as the lungs) induced by autoimmune arthritis contribute to increased breast cancer-associated bone and lung metastasis. We have used a recently established animal model of spontaneous autoimmune arthritis known as the SKG mice. The SKG mice is a mutant on the Balb/c background which spontaneously develop T cell-mediated chronic autoimmune arthritis as a consequence of a mutation of the gene encoding an SH2 domain of ZAP-70, a key signal transduction molecule in T cells (**8)**. This mutation impairs positive and negative selection of T cells in the thymus, leading to thymic production of arthritogenic autoimmune CD4+ T cells. The mice succumb to symmetrical joint swelling beginning in the small joints of the digits and progressing to larger joints, accompanied by severe synovitis with formation of pannus invading and eroding adjacent cartilage and subchondral bone. Genetic deficiency of IL-6, IL-1, or TNF-α inhibit development of arthritis in SKG mice (9), similar to the effects of anticytokine therapy in human arthritis (**10**). These clinical and immunopathological characteristics of arthritis in these mice make the strain a suitable model for testing our hypothesis. The SKG mice

were induced to develop mammary gland tumors by injecting syngeneic breast cancer cell lines, 4T1 (metastatic) or TUBO (non-metastatic) in the mammary fat pad. We found a three-fold increase in lung metastasis in the arthritic versus the non-arthritic mice and a significant increase in the incidence of osteolytic bone lesions in the arthritic mice. We identified the key pro-inflammatory factors that contribute to the increased incidence of breast cancer-associated secondary metastasis and to the severity of the bone disease. The finding provides a novel role of autoimmune arthritis and inflammation in promoting breast cancer associated metastasis to the site of inflammation.

RESULTS

Metastatic breast cancer cells contribute to the severity of arthritis in the SKG mice

SKG mice treated with one dose of zymosan A extract developed full-blown arthritis within 30-45 days with macroscopic evidence of joint swelling in the fingers and in the fore and hind limbs (as illustrated in Fig 1. c-f). Thirty-days post zymosan A injection, mice were challenged with $1x10^6$ syngeneic breast cancer cells (4T1 being metastatic or TUBO being non-metastatic) in the mammary fat pad. Age-matched SKG mice without zymosan injection were used as the pro-arthritic model, and Balb/c mice were used as the non-arthritic controls. Compared to the Balb/c control (Fig 1. a) and the pro-arthritic SKG (Fig 1. b) mice, the joint swelling was 5-fold and 2-fold higher in the SKG + zymosan + 4T1 (Fig 1. e and f) and SKG + zymosan + TUBO (Fig 1. c and d) mice respectively. More interestingly, the joints of the SKG + zymosan + 4T1 mice showed a 2-fold increase in swelling than the SKG + zymosan + TUBO mice (Fig 1. e and f compared to Fig 1. c and d) perhaps suggesting that the metastatic 4T1 breast cancer cells

augment the severity of arthritis. It was also clear that the non-metastatic TUBO cells did not augment the severity of macroscopic joint swelling in the SKG + zymosan mice since the severity of swelling in the SKG + zymosan + TUBO mice (Fig 1. c and d) was found to be identical to the SKG mice + zymosan mice that were not challenged with any tumor (data not shown). *Similar results were seen in N=10 mice per experimental group.*

It must be noted that 4T1 or TUBO-challenged SKG mice without zymosan did not show gross macroscopic evidence of joint swelling (data not shown), however, when hematoxylin & eosin (H&E) stained sections from the joints of 4T1-challenged SKG mice were examined, we observed severe inflammation as evidenced by the high degree of cellular infiltration into the bone joints (Fig 1. k). That however was not the case in the joints of TUBO-challenged SKG mice (Fig 1. i) providing further evidence that factors from the metastatic 4T1 breast cancer cells contribute to the severity of inflammation in the pro-arthritic SKG mice. Highest severity of synovial hyperplasia and erosion of articular cartilage and bone in the phalangeal joints of the hind limb resulting in severe cellular infiltration and inflammation was found in joints of the SKG + zymosan + 4T1 mice **(**Fig1. l**)**. As was expected, the SKG + zymosan + TUBO mice also showed synovial hyperplasia and erosion of articular cartilage and bone in the hind limb joints **(**Fig 1. j**)** but the severity of destruction was much less than evidenced with 4T1 challenge (Fig 1. l). The severity of inflammation in the TUBO challenged SKG + zymosan mice (Fig 1. j) was the same as SKG + zymosan mice without any tumor challenge (data not shown) suggesting that the non-metastatic TUBO cells did not enhance the severity of arthritis induced by zymosan. Inflammation was not observed in zymosan-treated Balb/c mice or in saline-treated SKG mice **(**Fig 1. g and h respectively**)**. Comparing the joint

infiltration in the SKG + 4T1 (Fig1. k) and SKG + TUBO (Fig1. i) mice, it becomes clear that the metastatic 4T1 breast cancer cells contribute to the vicious cycle of osteolytic destruction. *Several sections from N=10 mice per experimental group were examined with similar results. Sections of the distal hind limb joints are shown.*

Significantly higher primary breast tumor burden in pro-arthritic SKG mice as compared to the non arthritic Balb/c mice

Next we questioned 'if pro-arthritic or arthritic milieu affects the growth of primary breast tumors and secondary metastasis *in vivo*'. SKG mice challenged with 4T1 metastatic breast cancer cells showed significantly higher tumor burden in the mammary gland compared to the non arthritic Balb/c mice (Fig 1. m **$p<0.01$). However, to our great surprise, SKG mice that were treated with zymosan and challenged with 4T1 had significantly lower tumor burden as compared to the SKG mice without zymosan (*$p<0.05$). In fact, there was no difference in the primary 4T1 tumor burden between the SKG mice with zymosan and Balb/c control mice. Similar results were also observed with the non-metastatic TUBO cells (Fig 1. n) indicating that the tumor – type did not matter and that the innate immune responses elicited by zymosan injection regulate the primary tumor growth but not the secondary metastasis as discussed in the next section.

Three-fold increase in breast cancer-associated lung metastasis in pro-arthritic and arthritic mice augmented by severe inflammation in the lungs

More than three fold increase in lung metastasis was observed in the SKG mice challenged with the metastatic 4T1 breast cancer cells as compared to the non-arthritic Balb/c mice **(**Fig 2. g - i and Table 1**)** regardless of whether the SKG mice received the

zymosan treatment. One hundred percent of the SKG mice (8/8, Fig 2. j and Table 1. b) and 100% (9/9, Fig 2. j and Table 1. c) of the SKG + zymosan A mice developed lung metastasis with the 4T1 tumors whereas only 27% (3/11) of the non-arthritic Balb/c mice developed lung metastasis (Fig 2. j and Table 1. a). The number and size of the lung lesions were carefully evaluated for each mouse and were found to be significantly higher in the SKG versus the Balb/c mice **(**Table 1. a-c). It must be noted that this is true metastasis as the tumor cells were injected in the mammary fat pad and metastasis evaluated 4-weeks post tumor challenge. Lungs from TUBO-challenged mice show no metastatic lesions in the Balb/c or SKG mice albeit 2/8 mice in the SKG + zymosan group developed lung lesions (Fig 2. d - f, and j and Table 1. d). As was expected, there were no metastatic lesions in lungs of control Balb/c, SKG, and SKG-zymosan mice without tumor challenge (Fig 2. a - c, and j). Representative lungs from each experimental group are shown (Fig 2. a - i).

To investigate a mechanism of the increased lung metastasis in the pro-arthritic and arthritic mice, we first examined the lung histology. It became apparently clear that all lungs that developed metastasis were packed with interstitial inflammatory cellular infiltrate characterized by prominent neutrophilic and granulocytic cells and activated macrophages **(**Fig 2. m-v and Table 1). We graded the severity of lung infiltration and arbitrarily assigned levels of infiltration as ‘no’, ‘low’, ‘medium’, and ‘high’. Table 1 shows a correlation between the level of lung infiltration and severity of lung metastasis in all mice. Along with the cellular infiltrates, epithelial metastatic lesions were identified in the lungs as indicated by the solid yellow arrowheads (Fig 2. p, r and t). The infiltration was most prominent in the lungs of the SKG mice (+/- zymosan) bearing the

4T1 breast tumors (Fig 2.r and t Table 1. b-c**)**. These same lungs show severe lung metastasis (Fig 2. h-j and Table 1. b-c). It is interesting to note that although all lungs from the SKG + zymosan + TUBO group had low level infiltration, the two mice that develop lung metastasis were the only ones with higher levels of neutrophilic and granulocytic infiltration (Fig 2. p and Table 1. d). The lungs from SKG (Fig 2.u), SKG + zymosan (Fig 2. v, Balb/c + TUBO (Fig 2. m), SKG + TUBO (Fig 2. n, Balb/c + zymosan + TUBO (Fig 2. o), and Balb/c + zymosan + 4T1 (Fig 2. s) mice all show normal lung architecture which correlate with zero lung lesions (Table 1). The one mouse in Balb/c + 4T1 group that developed lung lesions also showed low-level cellular infiltrates (Fig 2. q and Table 1a). Thus, a high degree of correlation exists between cellular infiltrates in the lungs and lung metastasis (Table 1) suggesting a chemo-attractant microenvironment created in the lungs of pro-arthritic mice for recruiting and nurturing breast cancer cells to grow.

The next obvious question was whether the severity of lung metastasis follows the severity of arthritis? We compared the severity of metastatic lung lesions in the arthritic mice to the inflammation in the synovial membrane of the bone joints of the same mice to decipher the overall correlation between severity of arthritis and lung metastasis. A representative mouse from the SKG + 4T1 is shown in Fig 2. k and l in which severe inflammation in the fore limbs and hind limbs correlated with severe metastatic lung lesions. A statistical analysis for correlation coefficient was conducted which clearly indicated that severity of lung metastasis correlated with the severity of the inflammation in the bone microenvironment for the SKG + 4T1 and SKG + zymosan + 4T1 groups

(0.97 and 0.93 respectively) **(**Fig 2. w**).** With the TUBO-challenged mice, the analysis was not as clear since TUBO cells are normally non-metastatic (Fig 2. w).

Increased expression of pancytokeratin positive epithelial cells in the arthritic bones coupled with increased expression of CD31 and COX-2.

Since cellular infiltration in the lungs greatly facilitated the recruitment of breast cancer cells to the site (Fig 2.), we examined if high infiltration/inflammation in the bones of the arthritic mice had a similar effect. We have already shown that there is increased cellular infiltration in the bones of arthritic mice challenged with the 4T1 tumors **(**Fig 1. k and l**),** and that the bone infiltration correlated statistically with the severity of lung metastasis **(**Fig 2. j, k, l and w**).** We examined the levels of pro-inflammatory factors in the bone that may create a milieu conducive for increased bone metastasis. Inflammation in the joints is usually represented by high levels of cyclooxygenase-2 (COX-2) expression which plays a critical role in fostering tumor growth. In the same context, increased platelet endothelial cell adhesion molecule (CD31) expression in the bone correlates with tumor cell homing and spreading. In comparison to the Balb/c mice, there was increased expression of COX-2 and CD31 in the bones of the arthritic mice challenged with the 4T1 tumors (compare Fig 3 a and c with b and d for COX-2 and Fig 3. g and h with i and j for CD31), more so than in the arthritic mice with no tumor challenge **(**compare Fig 3. e and f with b and d for COX-2 and k and l with i and j for CD31) or in bones of SKG mice challenged with the non-metastatic TUBO cells **(**data not shown**).** The results imply that factors from the metastatic 4T1 tumors enhances the severity of inflammation in the bone and thus creates a favorable microenvironment for tumor cell homing and growth. To directly test the presence of epithelial (tumor) cells in the inflamed bone, sections of the

bone were stained with pan-cytokeratin antibody that specifically stains epithelial cells. Increased expression of pancytokeratin-positive areas was only evident in the bones of the arthritic mice challenged with the 4T1 tumors (Fig 3. n and p). Bones from all other experimental groups were negative (Fig 3. m, o, q, and r)**.** Brown staining represents CD31, COX-2, and pancytokeratin positivity. *Bones from n=10 mice were examined with similar staining patterns.*

Increased 4T1 bone lesions in the arthritic versus non-arthritic mice

The bone from n = 4 - 5 mice were analyzed by Faxitron imaging. Representative images are shown in Fig 3. s - x. Osteolytic and/or sclerotic bone lesions were determined in ~50% (2/4) of SKG mice and 33% (2/5) of the SKG + zymosan mice challenged with the 4T1 metastatic tumor cells **(**Fig 3. v and w**)**. These bones show distinct radiolucencies in the distal femoral diametaphysis indicating apparent osteolytic bone lesion (as indicated by the arrows). In contrast, none of the Balb/c mice challenged with 4T1 tumors developed bone lesions (Fig 3. t) nor did SKG mice challenged with TUBO cells (Fig 3. s, u, and x). The results indicate that bone lesions only occur in the pro-arthritic bones but not in non-arthritic bones (Fig 3. y).

M-CSF, IL-6, IL-17, TNF-α and VegF are the underlying factors for increased metastasis=associated with 4T1 tumors in the arthritic mice.

To determine the possible mechanism that drives the 4T1 cells to become more metastatic in the arthritic model, we evaluated the circulating levels of pro-inflammatory cytokines and chemokines in the sera of the arthritic versus non-arthritic mice. A custom mouse cytokine array was designed to test the sera for the presence of 10 cytokines

known to be associated with AA, inflammation, and metastasis. These included macrophage colony stimulating factor (M-CSF), tumor necrosis factor-alpha (TNF-α), Interferon-gamma (IFN-γ), VegF, Interleukin-17 (IL-17), matrix metalloproteinase-2 (MMP-2), Interleukin-6 (IL-6), Insulin-like growth factor-II (IGF-II), Interleukin-1beta (IL-1β), and Interleukin-4 (IL-4) **(Fig 4. g)**. Significant increases in levels of M-CSF, IL-17 (1.5 fold), TNF-α, VegF and IL-6 (1.2 fold) was noted in SKG mice challenged with the 4T1 tumors as compared to SKG mice challenged with TUBO tumors (compare Fig 4. b with e). A graphical representation of the values from the densitometric analysis is provided in Fig 4. h which clearly show the increase. SKG mice with zymosan show similar results as the SKG mice without zymosan (Fig 4. c, f and h). In contrast, M-CSF was the only cytokine that was upregulated in the 4T1 challenged Balb/c mice versus TUBO-challenged mice (Fig 4. a, d and h). Thus, increase in the M-CSF level seems to be a function of the 4T1 breast tumors regardless of the arthritic milieu, whereas increases in the IL-17, IL-6, TNF-α, and VegF levels were certainly driven by a combination of the metastatic 4T1 tumors and AA but not by either condition alone. SKG and Balb/c mice that were not challenged with any tumor showed similar cytokine levels as TUBO-challenged mice (data not shown).

DISCUSSION

The main goal of this study was to determine if inflammation in the bone and other organs caused by AA supports secondary metastasis associated with breast cancer. We establish that the chronic inflammation in the bone and lung caused by AA and the subsequent increase in circulating levels of proinflammatory cytokines may be the

underlying mechanism contributing to the increased metastasis (schematically represented in Fig 5. i)**.**

Results from the preclinical studies clearly indicate that breast cancer associated metastasis to the lung and bone is increased in the arthritis mice versus the non-arthritic mice (**Fig 2 and 3**). In addition, the metastatic breast cancer cells accentuate the severity of bone inflammation in the arthritic mice (**Fig 1**). This is partly explained by the fact that breast tumor cells produce factors that directly or indirectly induce the formation of osteoclasts. In turn, bone resorption by osteoclasts releases growth factors from the bone matrix that stimulate primary tumor growth, and bone destruction. This reciprocal interaction between breast-cancer cells and the bone microenvironment results in a vicious circle that increases both bone destruction and the tumor burden (17). The fact that the primary tumor burden is not increased in the SKG mice injected with zymosan (**Fig 1m and n**) can be explained by the fact that zymosan, a crude yeast cell wall extract activates the arthritogenic T cells through stimulating the innate immune response (11), which may help keep the primary tumor in check but does not reduce the incidence of metastasis.

We report that chronic inflammatory milieu and osteoclastic bone resorption caused by autoimmune arthritis influences the recruitment, retention, and proliferation of tumor cells not only to the bone (**Fig 3**) but also to the lungs **(Fig 2**). In fact a three fold increase in lung metastasis was observed in the arthritic mice challenged with the 4T1 breast cancer cells regardless of whether the SKG mice received zymosan A (**Fig 2 and Table I**). The lungs of arthritic mice with the 4T1 metastatic lesions express exceptionally high levels of interstitial inflammatory cellular infiltrates characterized by prominent

neutrophils, mast cells, activated macrophages, and possibly eosinophils (**Fig 2.r and t**). A statistical correlation was also observed between the severity of inflammation in the bone and the incidence of metastatic lung lesions (**Fig 2. w and Table 1**). Although it has been established that pro-inflammatory leukocytes alone can facilitate tumor cell extravasation and promote metastasis (18), this is the first study that establishes a correlation between the pro-inflammatory cell recruitment in the lungs during arthritis and the homing of the circulating cancer cells in the highly inflamed lungs (Figure 2 and Table 1).

Inflammation is a critical component of tumor progression and metastasis as well as arthritis (12,13,14, and 15). Many processes that occur during arthritis also occur during tumorigenesis. There is uncontrollable growth and inflammation in both; there is increased vascularity in both; and there are common cytokines and growth factors that are regulated in both. Many cancers including breast cancer may arise from sites of infection, chronic irritation, and inflammation. The tumor microenvironment, which is largely orchestrated by inflammatory cells, is critical in the neoplastic process, fostering proliferation, survival and migration (16). It is indeed interesting that post-menopausal women who are usually prone to developing some form of AA including osteoarthritis or inflammatory polyarthritis are also most likely to get breast cancer. Thus, it is not unlikely that the two diseases co-exist in these women. Our study begins to evaluate whether these two disease states molecularly interact and feed of each other.

Two of the pro-inflammatory factors that may contribute to the metastatic dissemination of breast cancer cells are COX-2 and VegF, both of which are activated during arthritis and breast cancer (25). We observe overexpression of COX-2 and VegF in the 4T1 tumors in the SKG mice with or without zymosan as compared to the tumors in the non-arthritic Balb/c mice (**data not shown**). Higher expression of COX-2 and VegF in tumors of the arthritic mice may indicate resistance to programmed cell death as well as increased cell migration, proliferation, and angiogenesis (19, 20, 21, and 22). High expression of VegF in AA is usually in response to high TNF-α (**Fig 4**), increasing endothelial permeability and swelling and also stimulating angiogenesis in the joints. The arthritic bone milieu is therefore comprised of inflammatory cells, cytokines, chemokines, cyclooxygenases (COX), lipooxygenase (LOX), and various eicosanoids, that may attract and foster tumor cells to the inflamed site (25). Our data goes a step further to suggest that the increased CD31 and COX-2 expression in the bones of arthritic SKG mice is promoted by metastatic breast tumor cells (**Fig 3. a-f and g-l**). Faxitron imaging of the bones suggested apparent osteolytic bone lesions only in the arthritic SKG mice challenged with 4T1 cells (**Fig 4. v and w**) which was corroborated by the presence of pancytokeratin (epithelial cell) positive patches (**Fig 3. n and p**).

As in human AA, cytokines play an essential role in the development of arthritis in the SKG mice (9). Several cytokines have been implicated in the mechanism of synovial cell activation and joint destruction in AA (27). In our study, serum analysis of cytokine proteins revealed higher expression of M-CSF, IL-17, IL-6, TNF-α, and VegF in the arthritic mice only when challenged with 4T1 metastatic breast cancer cells (**Fig 4)**. Elevated serum M-CSF predicts reduced survival in metastatic breast cancer patients. The

M-CSF produced by breast cancer cells and surrounding stromal cells increases osteoclast formation and maturation and enhances the expression of stromal RANK ligand, both of which increase osteolytic bone degradation (33). IL-17 has been identified as a crucial cytokine for osteoclastic bone resorption in AA patients. IL-17 acts on osteoblasts by stimulating COX-2–dependent prostaglandin E_2 (PGE_2), and osteoclast differentiation factor which differentiates osteoclast progenitors into mature osteoclasts, causing bone resorption. PGE_2 interacts with its eicosanoid receptors to induce the damage (26). IL-6 is an autocrine and paracrine growth factor for several cancers, including breast cancer (29) and stimulates cancer cell growth and contributes to recurrence and metastasis in breast cancer (30). VegF is known to play a critical role in vasculogenesis, angiogenesis, and metastasis. Upregulation of these cytokines may therefore account for the enhanced breast cancer associated secondary metastasis as well as accentuating the severity of arthritis in the SKG mice. Thus, blocking these pro-inflammatory cytokines may hold promise in reducing the severity of both diseases.

In conclusion, our data for the first time, implicate that chronic inflammation in the bone and lungs caused by AA creates a milieu that attract metastatic breast tumor cells to home and proliferate in those inflamed sites. These studies may have important clinical implications, especially in the prevention of secondary metastasis, in designing combination drug regimens, and as a diagnostic risk-assessment tool.

MATERIALS AND METHODS

Mice

SKG mice have been established from the closed breeding colony of Balb/c mice (8). Two sets of SKG breeding pairs were purchased from CLEA International Japan and were breeded in our animal facility. All protocols were approved by the Mayo Clinic Internal Animal Care Review Committee.

Induction of Arthritis

The SKG mice fail to develop detectable arthritis in a microbially clean environment, despite their thymic production of arthritogenic autoimmune T cells that persist in the periphery. However, zymosan A, a crude yeast cell wall extract, can provoke arthritis in SKG mice (**11).** Two month old mice were given a single intraperitoneal (ip) injection of 2mg of zymosan A in 100ul of 0.15M NaCl per mouse and joint swelling was macroscopically examined starting at 14-days post zymosan A treatment. Thirty days post zymosan A, >95% of the mice develop polyarthritis in small and large joints. At 30-days post zymosan A injection, mice were challenged with $1x10^6$ syngeneic breast cancer cells (4T1: metastatic or TUBO: non-metastatic) in the mammary fat pad. Age-matched SKG mice without zymosan A injection were used as the pro-arthritic model, and Balb/c mice were used as the non-arthritic controls. Tumors were allowed to grow for 4 weeks. Zymosan A was purchased from Sigma-Aldrich, USA. A 1% solution of zymosan was made in 0.15 M sodium chloride (NaCl), placed in boiling water bath for one hour, centrifuged for 30 minutes at 4000 rpm, supernatant discarded, and the residue suspended evenly in the 0.15M NaCl to the desired concentration. It is established that the glucose polymer B-1,3-D-glucans (B-glucans), the main constituents of zymosan A, are responsible for the arthritogenic effect **(11)**.

Cell Culture

The 4T1 mammary carcinoma cell line was purchased from The American Type Cell Culture Collection and the TUBO mammary carcinoma cell line was generously provided by Dr Joseph Lustgurten, Mayo Clinic College of Medicine. 4T1 and TUBO cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 1% Glutamax-1 and 1% penicillin-streptomycin. Cells were maintained at log phase at 37^0C with 5% carbon dioxide. 4T1 is a highly metastatic breast cancer cell line derived from a spontaneously arising BALB/c mammary tumor. TUBO is a cloned cell line established from a mammary carcinoma of BALB-neuT mice. TUBO is considered to be a non metastatic cell line.

Induction of Tumor

One million 4T1 and TUBO cells were injected directly into the mammary fat pad of female arthritic SKG and non-arthritic Balb/c mice. The tumors were visible in the mammary fat pad in 5-7 days. The tumors were allowed to grow for 4 weeks and animals sacrificed thereafter.

Measurement of cytokines using Custom Mouse Cytokine/Chemokine Antibody Array kit

The RayBio® Custom Mouse Cytokines Antibody Array kit was purchased from RayBiotech (Norcross, GA) and used according to the manufacturer's instructions. Briefly, after blocking, membranes were incubated for 1.45 h with the experimental serum. The membranes were washed and incubated with biotin-conjugated antibodies for 1.45 h. The membranes were washed again and incubated with streptavidin-conjugated

horseradish peroxidase (HRP) for 2 h, washed, and developed using an enhanced chemiluminescent substrate for HRP. Chemiluminescence was detected using a EpiChemi3® Darkroom imaging system and LabWorks® densitometry software (both from UVP Bioimaging, Upland, CA). Data was corrected for background signal and normalized to positive controls using RayBio® Analysis Tool software.

Histologic and immunohistochemical staining

Lungs and a part of the tumor were formalin fixed in 10% neutral-buffered formalin (pH 6.8-7.2) for a minimum of 24 hours. Paraffin embedded blocks was prepared by the Histology Core at The Mayo Clinic and 4-micron thick sections were cut for hematoxylin eosin (H&E) staining and for immuno-staining. For bones: The fore limb and hind limb were dissected from the mice and immersed in 10% neutral-buffered formalin (pH 6.8-7.2) overnight. For decalcification, Cal-Rite (Richard Allan Scientific, Kalamazoo, MI), a formic acid decalcification agent was used for ~72 hours followed by the conventional processing method. To determine CD31, COX-2 and Pancytokeratin expression in the bones, COX-2, CD-31, and Pancytokeratin (Santa Cruz Biotechnologies) antibodies were used at 1:50 and incubated overnight at 4◦C followed by the DAKO anti-goat secondary (1:100 dilution) for 45 minutes at RT for both COX-2 and CD-31. For Pancytokeratin staining, DAKO anti-mouse secondary was used at 1:100 for 45mins at RT. 3, 3" –Diaminobenzidine was used as the chromogen and hematoxylin was used as counterstain. Slides were examined under light microscopy and pictures taken at 200X magnification.

Statistical analysis:

Student’s t-test was used for comparing the level of significance between the experimental groups. Correlation Coefficient was determined using the JMP statistical program.

Table 1

	Group	Mice #	Infiltrating cells in the lungs (High/Medium/Low)	Size of Lung lesions			# Lung lesions
(a)				Large	Medium	Small	Total
	Balb/c+4T1(3/11)	1	No	0	0	0	0
		2	No	0	0	0	0
		3	No	0	0	0	0
		4	No	0	0	0	0
		5	No	0	0	0	0
		6	Low	8	0	9	17
		7	No	1	0	0	1
		8	No	0	0	0	0
		9	No	0	0	0	0
		10	No	0	0	0	0
		11	No	0	0	2	2
(b)	**SKG+4T1(8/8)**	1	High	16	0	9	25
		2	High	10	0	11	21
		3	High	0	22	0	22
		4	High	15	0	5	20
		5	High	2	0	10	12
		6	High	0	0	13	13
		7	Medium	0	0	9	9

		8	High	0	0	14	14
(c)	**SKG+zymosan+4T1(9/9)**	1	Medium	0	3	0	3
		2	Medium	2	0	4	6
		3	High	10	0	9	19
		4	Low	0	0	0	3
		5	Medium	0	0	0	5
		6	Medium	0	0	5	5
		7	Medium	0	1	7	8
		8	High	9	0	9	18
		9	High	0	10	6	16
(d)	**Balb/c+Tubo(0/7)**	No lesions	No	0	0	0	0
	SKG+Tubo(0/10)	No ;esions	No	0	0	0	0
	SKG+zymosan+Tubo(2/8)	1	Low	0	0	0	0
		2	Low	0	0	0	0
		3	Low	0	0	0	0
		4	Low	0	0	0	0
		5	Low	0	0	0	0
		6	Low	0	0	0	0
		7	Medium	0	10	0	10
		8	Medium	0	4	0	4

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REFERENCES

8. deVisser,K.E.,and M.Coussens. 2006. The inflammatory tumor microenvironment and its impact on cancer development. *Contrib Microbiol.* 13:118-137**.**

9. Ziegler, J. 1998. Cancer and arthritis share underlying processes. *J Natl Cancer Inst* .90:802-803.

10. Franklin, J., M. Lunt, D. Bunn, D. Symmons, and A. Silman. 2007. Influence of inflammatory polyarthritis on cancer incidence and survival: results from a community-based prospective study. *Arthritis Rheum*. 56:790-798.

11. Mellemkjaer, L., M.S. Linet, G.Gridley, M.Frisch, H. Moller, and J.H. Olsen. 1996. Rheumatoid arthritis and cancer risk.. *Eur J Cancer*. 32: 1753-1757.

12. Askling,J., C.M. Fored, L. Brandt , E. Baecklund, L. Bertilsson, N. Feltelius et al. 2005. Risks of solid cancers in patients with rheumatoid arthritis and after treatment with tumor necrosis factor antagonists. *Ann Rheum Dis* . 64:1421-1426.

13. Askling, J., C.M.Fored, E. Baecklund, L.Brandt , C. Backlin , A. Ekbom , et al. 2005. Haematopoietic malignancies in rheumatoid arthritis: lymphoma risk and characteristics after exposure to tumor necrosis factor antagonists. *Ann Rheum Dis* 64:1414-1420.

14. Andy J. Minn., P.G. Gupta, P. M. Siegel, P. D. Bos, W. Shu, D. Giri, A. Viale, A. B. Olshen, W. L. Gerald and J.Massague.2005. Genes that mediate breast cancer metastasis to lung. *Nature* 436, 518-524.

15. Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S et al. 2003.Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature* . 426: 454–60 ,.

16. Hata, H., N. Sakaguchi, H. Yoshitomi, Y. Iwakura, K,Sekikawa, Y. Azuma, C.Kanai, E. Moriizumi, T. Nomura, T.Nakamura, and S. Sakaguchi.2004. Distinct contribution of IL-6,TNF-alpha, IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J.Clin. Invest*. 114:582-588.

17. Firestein,G.S. 2003. Evolving concepts of rheumatoid arthritis. *Nature*. 423:356-361

18. Hiroyuki,Y., N. Sakaguchi,K.Kobayashi, G.D. Brown, T.Tagami, T.Sakihama, K. Hirota, S. Tanaka, T. Nomura, I. Miki, S. Gordon, S.Akira, T.Nakamura, and S. Sakaguchi. 2005. A role for fungal β-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J Exp Med*. 201: 949–960.

19. Ziegler, J. 1998. Cancer and arthritis share underlying processes. *J Natl Cancer Inst* . 90:802-803.

20. Coussens, L. M., and Z. Werb. 2002. Inflammation and cancer. *Nature.* 420:860-867.

21. Philip, M., D. A. Rowley, and H. Schreiber. 2004. Inflammation as a tumor promoter in cancer induction. *Semin Cancer Biol* 14:433-439.

22. Clevers, H. 2004. At the crossroads of inflammation and cancer. *Cell* 118:671-674.

23. Balkwill, F., K. A. Charles, and A. Mantovani. 2005. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 7:211-217.

24. David ,G.R. 2004. Mechanisms of bone metastasis.*N Eng J Med*; 350:1655-64.

25. Slattery,M.J.S., S.Liang, and Dong. 2005. Distinct role of hydrodynamic shear in leukocyte-facilitated tumor cell extravasation. *Am J Cell Physiol*. 288: 831-9.

26. Folkman, J. 1989.What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst*. 82:4–6.

27. Risau, W. 1997.Mechanisms of angiogenesis. *Nature.* 386:671–674.

28. Folkman, J. 1995.Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med*. 1:27–31.

29. Gimbrone, M., S. Leapman , R. Cotran, and F.Judah. 1972. Tumor dormancy in vivo by prevention of neovascularization. *J Exp Med*.;136:261–276.

30. Linderholm ,B., B. Lindh , B. Tavelin , K. Grankvist, and R. Henriksson .2008. p53 and Vascular-Endothelial-Growth-Factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. *Int J Cancer*.89:51–62.

31. Gasparini,G.2000. Prognostic value of Vascular Endothelial Growth Factor in breast cancer. *Oncologist*. 10:37–44.

32. Yong,M.Z., M.N.S. Azahri, C.Y.Danny, and J.W. Penella. 2008. Effects of COX-2 inhibition on expression of vascular endothelial growth factor and interleukin-8 in lung cancer cells.*BMC Cancer*. 2008; 8: 218

33. Kotake, S., N. Udagawa, N. Takahashi, K. Matsuzaki, K. Itoh, S. Ishiyama, S. Saito, K. Inoue, N. Kamatani, M. T. Gillespie, T. J. Martin, and T. Suda. 1999. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest* 103:1345-1352.

34. Arend, W.P., and J.M. Dayer.1995. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor alpha in rheumatoid arthritis. *Arthritis Rheum.* 38:151–60.

35. Debra,E.L., R.N. Nancy, L. McCain, and J. Walter. 2008. Cytokine Comparisons Between Women With Breast Cancer and Women With a Negative Breast Biopsy. *Nurs Res.*. 57(1)51-58.

36. Ben-Baruch, A. 2006. Inflammation-associated immune suppression in cancer: The roles played by cytokines, chemokines and additional mediators. *Seminars in Cancer Biology*. 161:38–52.

37. Leek, R., and A. Harris. 2002.Tumor-associated macrophages in breast cancer. *Journal of Mammary Gland Biology and Neoplasia*. 7:177–189.

38. Numasaki, M., J.Fukushi, M.Ono , S.K.Narula, P.J.Zavodny, T. Kudo, P. D. Robbins**,** H. Tahara**,** and M. T. Lotze**.** 2003 Interleukin-17 promotes angiogenesis and tumor growth. *Blood*. 101:2620–2627.

39. Alexandrakis, M.G., C.A.Pappa, S.Miyakis, A.Sfiridaki , M.Kafousi , A.Alegakis, and E. Stathopoulose. 2006. Serum interleukin-17 and its relationship to angiogenic factors in multiple myeloma. *European Journal of Internal Medicine*. 17:412–416.

40. K. Leitzel, Ettenberg.S, Walsh.R, Abraham.J, Modur.V, Braendle.E, Evans.D.B, Ali.S.M, Demers.L and A. Lipton. 2007. Elevated serum M-CSF level predicts reduced survival in metastatic breast cancer patients. *Journal of clinical oncology*.2007 ASCO Annual Meeting Proceedings.Vol 25, No 18S(June 20 supplement),2007:10591.

FIGURE LEGENDS

Figure 1. **Induction of arthritis and tumor burden in SKG mice: a-f:** Images of the hind and fore limbs **a:** control Balb/c mice (no inflammation)**; b**: control SKG mice (no inflammation); **c and d:** SKG mice + zymosan A + TUBO (low-level inflammation); **e and f**: SKG mice + zymosan A + 4T1 cells (severe swelling). **g-l:** H&E staining of sections from the joints: **g:** Balb/c mice; **h:** SKG mice; **i:** SKG mice + TUBO cells; **j:** SKG mice + zymosan A + TUBO cells showing some erosion of articular cartilage; **k**: SKG mice + 4T1 cells showing severe inflammation in the phalangeal joints; and **l**: SKG mice + zymosan A + 4T1 cells showing severe synovial hyperplasia and erosion of articular cartilage and bone in phalangeal joints with severe inflammation; **o:** control SKG + zymosan without tumor challenge (moderate inflammation); **m-n:** Tumor burden

in grams (g) **m:** mice injected with 4T1 cells; **n:** mice injected with TUBO cells. Compared to the non-arthritic Balb/c mice, a significant increase in tumor burden was observed in SKG mice with 4T1 (**p<0.01) and TUBO cells (*p<0.05). SKG mice + zymosan A prior to tumor challenge showed similar primary tumor burden as control Balb/c mice. *Note: All tumor cells were injected in the mammary fat pad and sacrificed 4-weeks post tumor challenge.*

Figure 2. AA-related inflammation in the lungs is associated with a 3-fold increase in the number of lung metastatic lesions in the SKG mice. Representative images of lungs from **a:** Balb/c; **b:** SKG; **c:** SKG + zymosan A; **d:** Balb/c injected with TUBO cells; **e:** SKG without zymosan A + TUBO cells; **f:** SKG + zymosan A + TUBO cells (2/8 mice developed lung lesions); **g**: Balb/c + 4T1 cells (3/11 mice developed lung lesions); **h**: SKG without zymosan A + 4T1 cells (8/8 mice developed severe lung metastasis); **i:** SKG + zymosan A + 4T1 cells (9/9 mice developed severe lung metastasis). *Note: All tumor cells were injected in the mammary fat pad and sacrificed 4-weeks post tumor challenge.* **j:** Percent mice that developed lung metastasis **m-v:** H&E staining of the lung sections from **m:** Balb/c + TUBO; **n:** SKG + TUBO; **o:** Balb/c + zymosan A + TUBO; **p:** SKG + zymosan A + TUBO (high infiltration)**; q:** Balb/c + 4T1 (high infiltration due to the 4T1 cells, representative of the mice that had lung metastatic lesion); **r:** SKG + 4T1 (severe infiltration); **s:** Balb/c + zymosan A + 4T1 (no infiltration); **t:** SKG + zymosan A + 4T1 (severe infiltration); **u:** SKG (no infiltration); and **v:** SKG + zymosan A (no infiltration). **r and t:** The solid filled arrow represents lung metastatic lesions, unfilled arrow represents neutrophils and unfilled circles represent macrophages. **k and l:** Representative images from one SKG + 4T1 mice showing both

severe joint inflammation **(k)** as well as severe lung metastasis **(l). w:** A statistical analysis of correlation coefficient of 0.97 was observed between bone inflammation and lung metastasis in the SKG + 4T1 group with a 0.93 for the SKG + zymosan + 4T1 group. Correlation coefficient for the SKG + TUBO group was not calculated since there was no lung metastasis or bone inflammation noted in these mice. *All images taken at 200X magnification*

Figure 3. Increased expression of COX-2, CD31, and pancytokeratin in the bones of arthritic mice challenged with 4T1 cells. Immunohistochemical (IHC) staining for COX-2 (**a-f);** CD31 (**g-l);** and pancytokeratin **(m-r)** in bone sections from various experimental mice as indicated in the Figure. Increased expression of COX-2, CD31, and pancytokeratin is observed in the bones of arthritic mice challenged with 4T1 tumor cells as compared to the non-arthritic Balb/c mice. *Brown staining represents COX-2, CD31, and pancytokeratin positive staining. All images are taken at 200X magnification.* Representative Faxitron images of bones (**s-w)**. Balb/c + TUBO (**s**); Balb/c + 4T1 (**t**); SKG + TUBO (**u**); SKG + 4T1 **(v)** showing a few small radiolucencies in distal femoral diametaphysis indicating apparent osteolytic bone lesion; and SKG + zymosan A + 4T1 (**w)** showing a medium size poorly defined radiolucency in the distal femoral diametaphysis possibly due to osteolytic bone lesion. N=4-5 mice from each group were examined using the faxitron. No lytic or sclerotic lesions were observed in any other experimental group. **y:** Percent mice that developed bone metastasis.

Figure 4. Serum analysis of cytokines revealed upregulation of several cytokines in the arthritic mice.

a-f. Membrane blots representing circulating cytokine levels in various groups of mice. The serum from non-arthritic Balb/c and arthritic SKG injected with 4T1 and TUBO cells were tested on custom mouse cytokines antibody membrane array. The arthritic SKG + TUBO and non-arthritic Balb/c + TUBO were used as controls. **g**. Array template is shown. **h:** A graphical representation of the upregulated cytokines observed on the membrane blots based on densitometric analysis. Upregulation of M-CSF, TNF-alpha, IL-17, VegF, and IL-6 is observed and highlighted in gray on the template (**g**).

Figure 5: Schematic model representing the vicious interaction between metastatic breast tumors and the inflammatory microenvironment in the bone and lung due to AA: During arthritis, neutrophils, and macrophages infiltrate the bones and lungs causing high degree of inflammation which trigger the release of pro-inflammatory cytokines (such as IL-17, IL-6, TNF-α, and VegF) in the circulation. These cytokines act directly or indirectly on the primary breast tumor cells enhancing their metastatic ability. In turn, the metastatic breast tumor cells release more pro-survival/pro-inflammatory cytokines including IL-6, TNF-α, VegF, and M-CSF and invade the underlying extracellular matrix and circulate in the blood and lymphatic systems of the arthritic mice. The large amounts of inflammatory cells and pro-inflammatory cytokines in the bones and lungs attract the tumor cells to leave the circulation and enter the organs. In the bone, the tumor cells stimulate the release of IL-17 which differentiates osteoclast progenitors into mature osteoclasts, causing bone resorption. The damage to the bone matrix allows retention and growth of the tumor cells in the site. The tumor cells also stimulate the surrounding stromal cells to release of M-CSF which increases osteoclast formation and maturation which increases osteolytic bone degradation and creates a milieu suitable for tumor cells

to form metastatic lesions. In the lungs, the infiltrating cells and the tumor cells release M-CSF, TNF-α, IL-6 and VegF stimulating vasculogenesis, angiogenesis and tumor growth. The final distribution of metastasis reflects the relative abundance of inflammation in the organs. The subsequent increase in circulating levels of proinflammatory cytokines, in particular M-CSF, VegF, IL-6, IL-17, and TNF-α is therefore the underlying factors contributing to the increased metastasis.

TABLE 1: High cellular infiltration in the lungs correlates with an increase in the number of lung metastatic lesions in the arthritic mice.

Number of lung lesions in non-arthritic Balb/c **(a)** and arthritic mice **(b and c)** challenged with 4T1 cells **(a-c)** or TUBO cells **(d).** Size and number of lung lesions are shown along with level of infiltration in the same lungs. High degree of correlation exists between cellular infiltration and metastatic lung lesions.

Figure 1

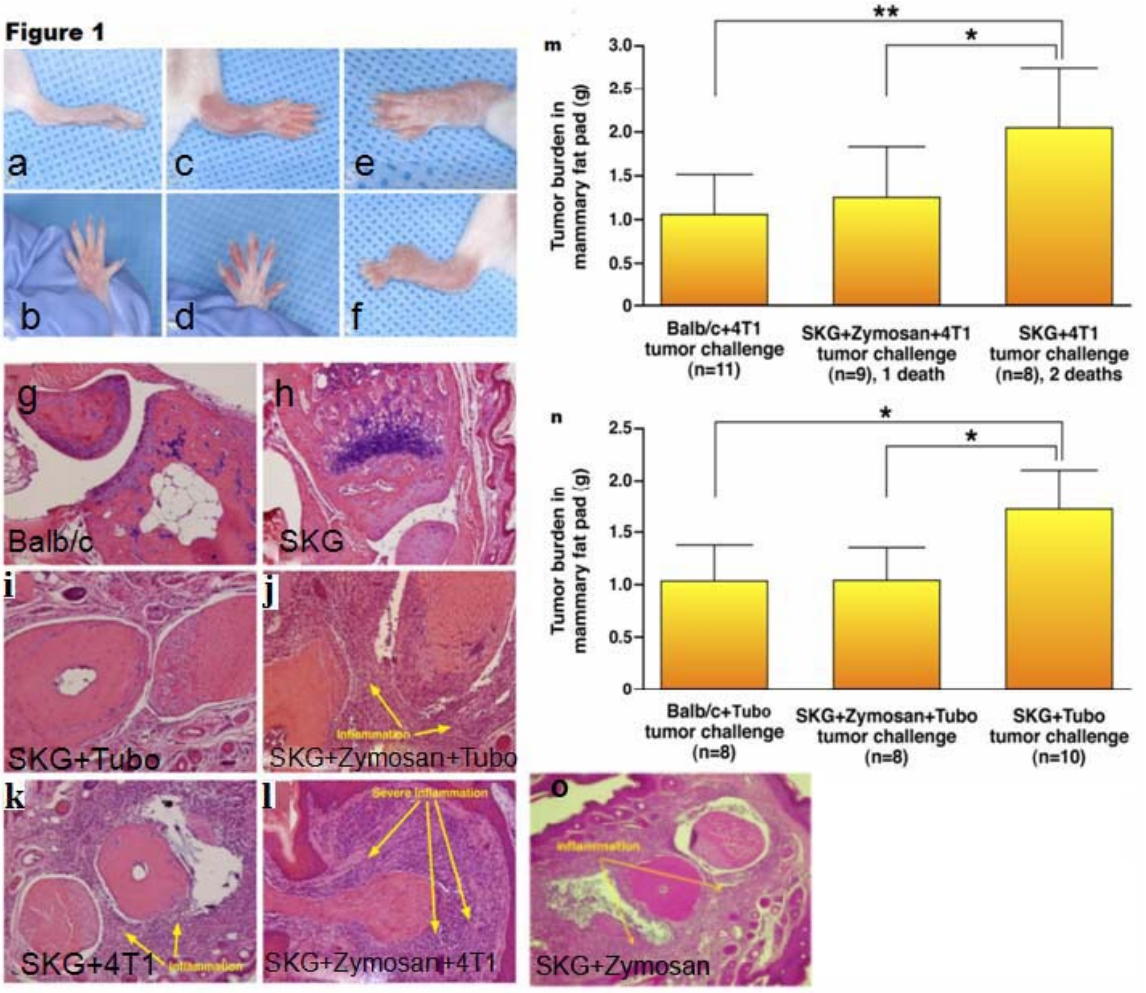

a
c
e
b
d
f
g
Balb/c
h
SKG
i
SKG+Tubo
j
Inflammation
SKG+Zymosan+Tubo
k
Inflammation
SKG+4T1
l
Severe Inflammation
SKG+Zymosan+4T1
m
**
*
Tumor burden in mammary fat pad (g)
3.0
2.5
2.0
1.5
1.0
0.5
0
Balb/c+4T1 tumor challenge (n=11)
SKG+Zymosan+4T1 tumor challenge (n=9), 1 death
SKG+4T1 tumor challenge (n=8), 2 deaths
n
*
*
Tumor burden in mammary fat pad (g)
2.5
2.0
1.5
1.0
0.5
0
Balb/c+Tubo tumor challenge (n=8)
SKG+Zymosan+Tubo tumor challenge (n=8)
SKG+Tubo tumor challenge (n=10)
o
Inflammation
SKG+Zymosan

Figure 2

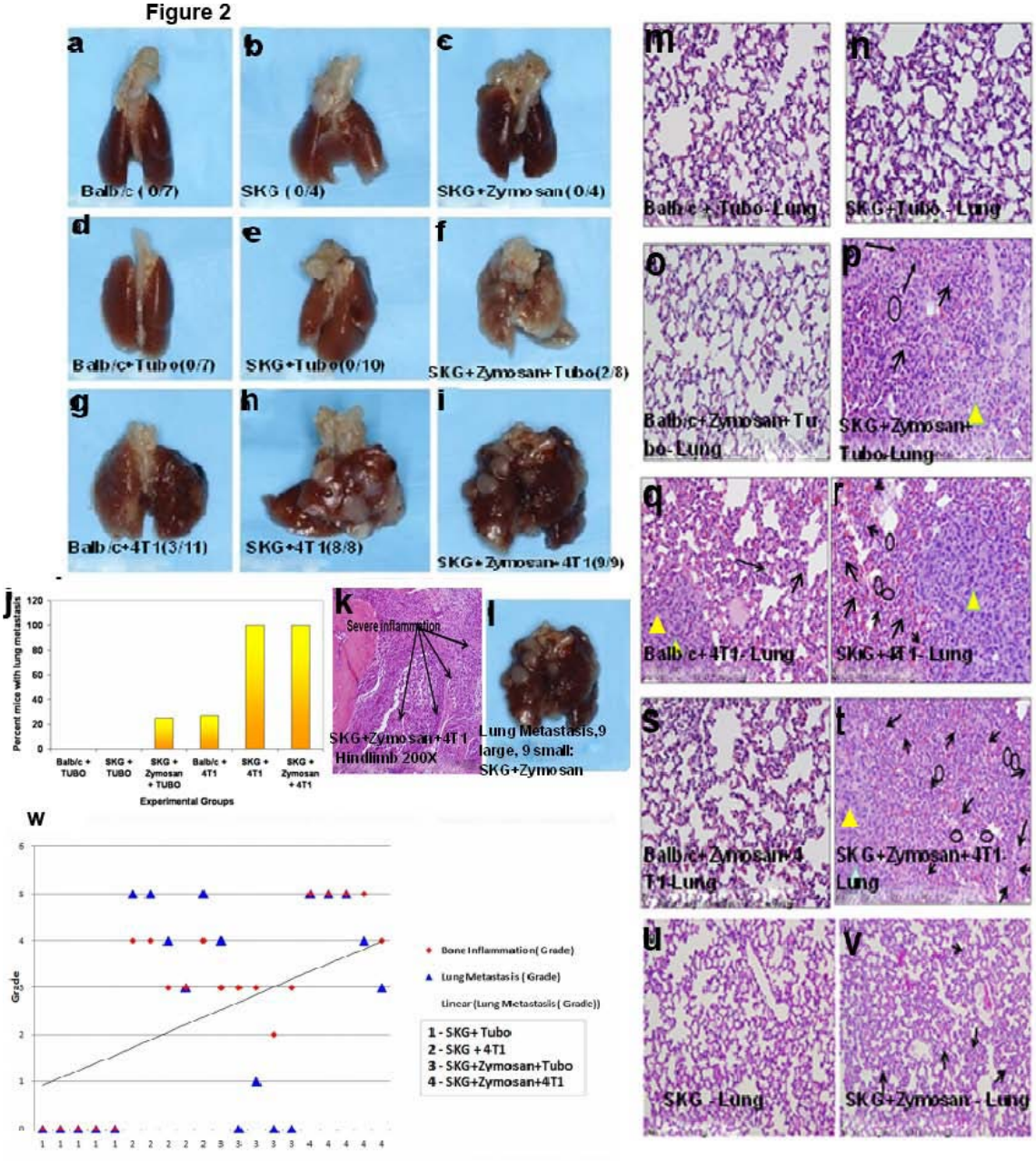

a
Balb/c (0/7)
b
SKG (0/4)
c
SKG+Zymosan (0/4)
d
Balb/c+Tubo(0/7)
e
SKG+Tubo(0/10)
f
SKG+Zymosan+Tubo(2/8)
g
Balb/c+4T1(3/11)
h
SKG+4T1(8/8)
i
SKG+Zymosan+4T1(9/9)
m
Balb/c+Tubo-Lung
n
SKG+Tubo - Lung
o
Balb/c+Zymosan+Tubo-Lung
p
SKG+Zymosan+Tubo-Lung
q
Balb/c+4T1- Lung
r
SKG+4T1- Lung
s
Balb/c+Zymosan+4T1-Lung
t
SKG+Zymosan+4T1-Lung
u
SKG - Lung
v
SKG+Zymosan - Lung
j
Percent mice with lung metastasis
Balb/c + TUBO
SKG + TUBO
SKG + Zymosan + TUBO
Balb/c + 4T1
SKG + 4T1
SKG + Zymosan + 4T1
Experimental Groups
k
Severe inflammation
SKG+Zymosan+4T1 Hindlimb 200X
l
Lung Metastasis,9 large, 9 small: SKG+Zymosan
w
Grade
Bone Inflammation (Grade)
Lung Metastasis (Grade)
Linear (Lung Metastasis (Grade))
1 - SKG+Tubo
2 - SKG + 4T1
3 - SKG+Zymosan+Tubo
4 - SKG+Zymosan+4T1

Figure 3

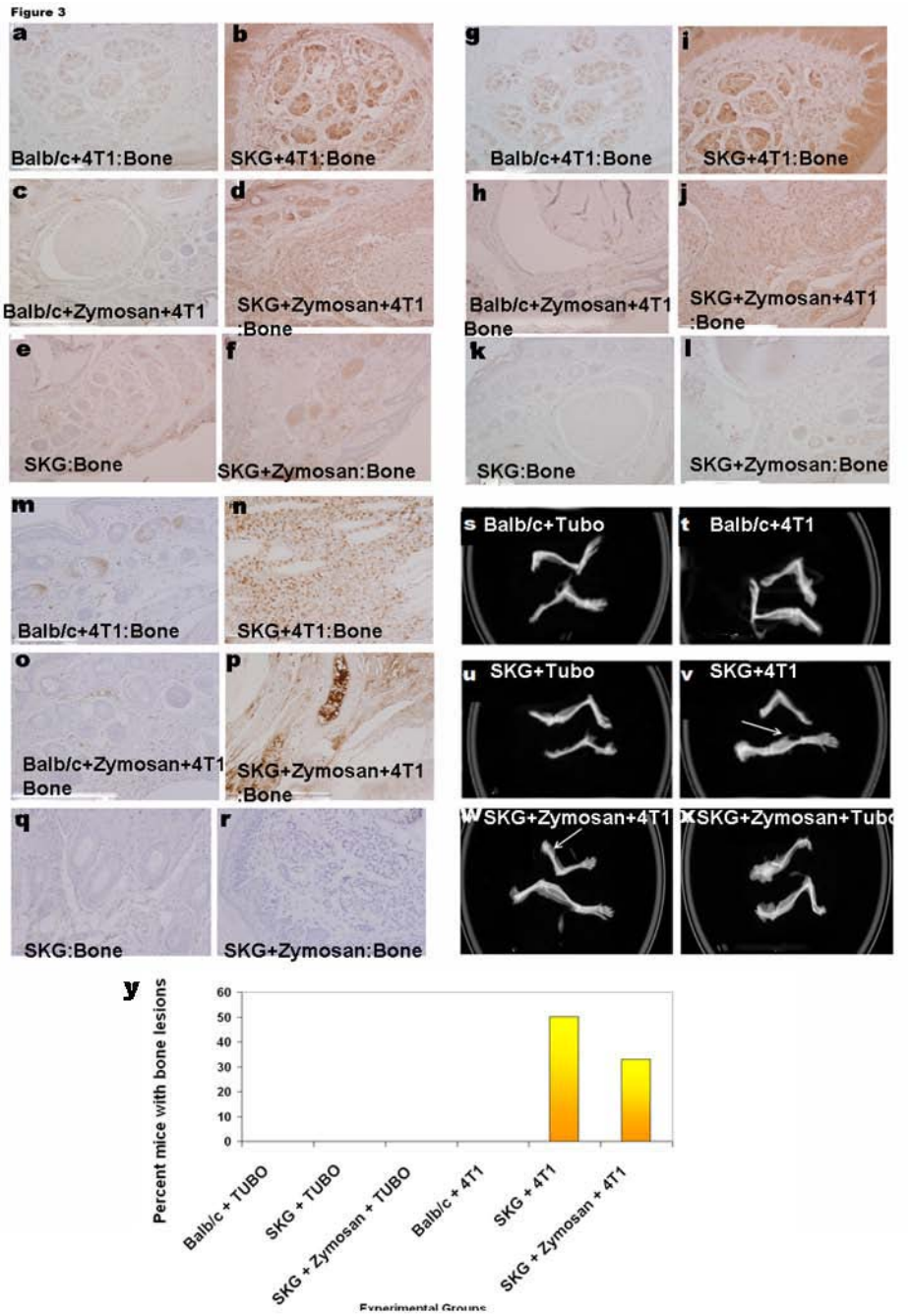
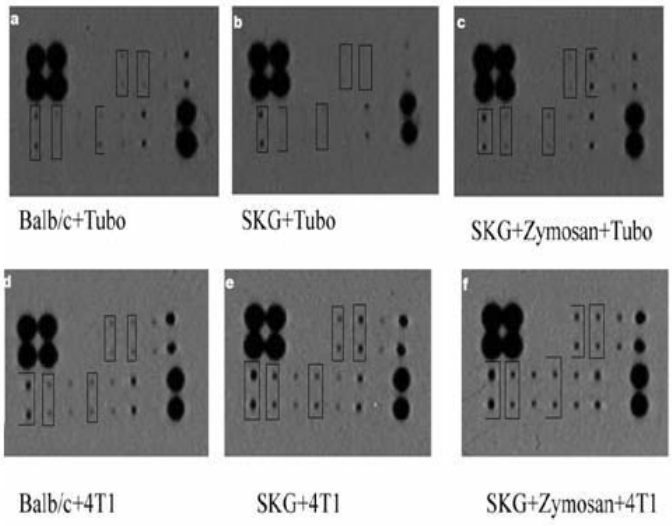

a
Balb/c+4T1:Bone
b
SKG+4T1:Bone
g
Balb/c+4T1:Bone
i
SKG+4T1:Bone
c
Balb/c+Zymosan+4T1
d
SKG+Zymosan+4T1 :Bone
h
Balb/c+Zymosan+4T1 Bone
j
SKG+Zymosan+4T1 :Bone
e
SKG:Bone
f
SKG+Zymosan:Bone
k
SKG:Bone
l
SKG+Zymosan:Bone
m
Balb/c+4T1:Bone
n
SKG+4T1:Bone
s Balb/c+Tubo
t Balb/c+4T1
o
Balb/c+Zymosan+4T1 Bone
p
SKG+Zymosan+4T1 :Bone
u SKG+Tubo
v SKG+4T1
q
SKG:Bone
r
SKG+Zymosan:Bone
w SKG+Zymosan+4T1
x SKG+Zymosan+Tubo
y
Percent mice with bone lesions
60
50
40
30
20
10
0
Balb/c + TUBO
SKG + TUBO
SKG + Zymosan + TUBO
Balb/c + 4T1
SKG + 4T1
SKG + Zymosan + 4T1
Experimental Groups

Figure 4



g

RayBio® Cytokine Antibody Arrays – Custom Array

	A	B	C	D	E	F	G	H
1	Pos	Pos	Neg	Neg	IL-17	IL-6	IL-1beta	IL-4
2	Pos	Pos	Neg	Neg	IL-17	IL-6	IL-1beta	IL-4
3	M-CSF	TNF-alpha	IFN-gamma	VEGF	MMP-2	IGF-II	BLANK	Pos
4	M-CSF	TNF-alpha	IFN-gamma	VEGF	MMP-2	IGF-II	BLANK	Pos

(h)

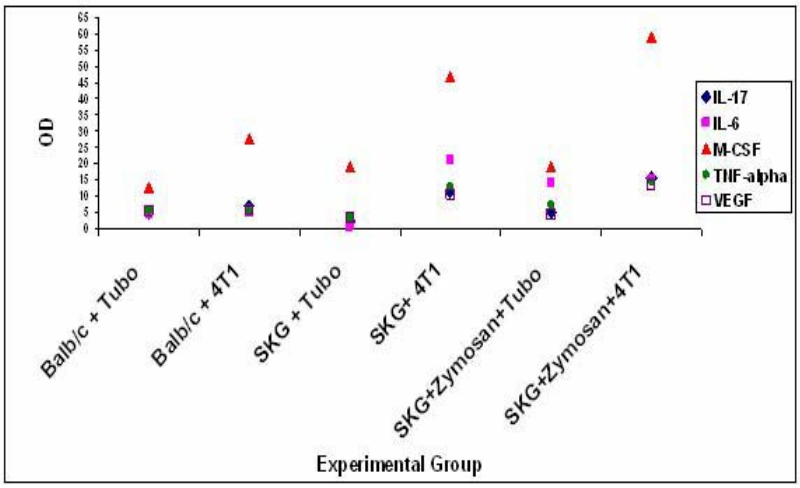
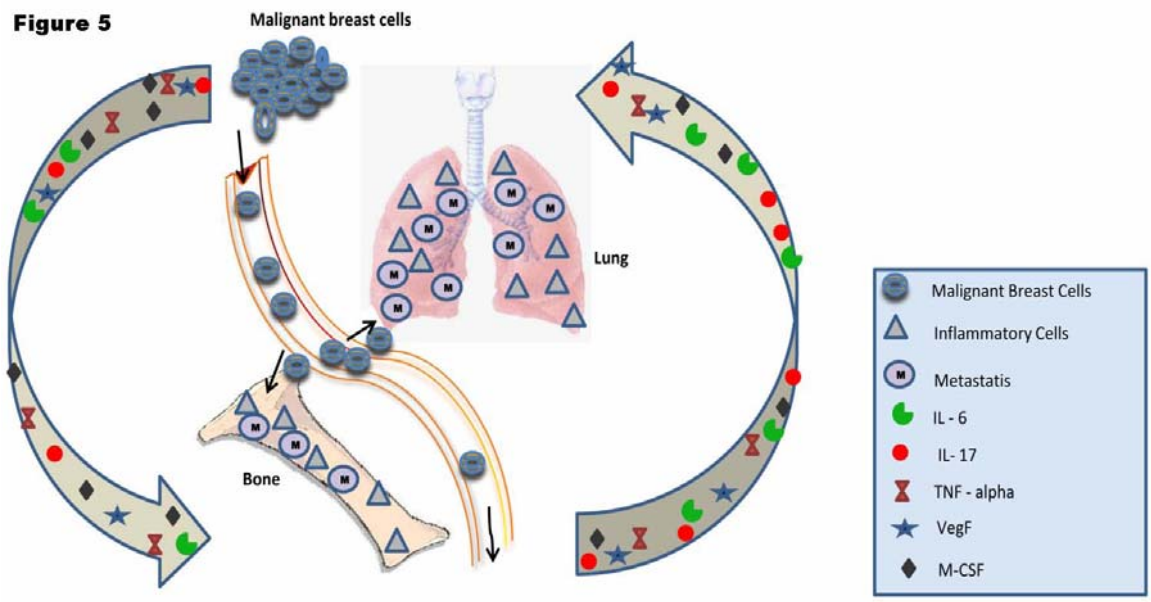


Figure 5


Malignant breast cells
Lung
Bone
Malignant Breast Cells
Inflammatory Cells
Metastatis
IL - 6
IL- 17
TNF - alpha
VegF
M-CSF